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Original Article

Activation of SRY accounts for male-specific hepatocarcinogenesis: Implication in gender disparity of hepatocellular carcinoma☆

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ABSTRACT

Sex affects the risk, treatment responses and outcome of many types of cancers. The mechanism of gender disparity in development of hepatocellular carcinoma (HCC) remains obscure. Sex-determining region on Y chromosome (SRY) was overexpressed in approximate 84% male patient HCC. Moreover, we are the first to generate a liver-specific transgenic (TG) murine model with overexpression of the male specific gene SRY. Subject to a single intraperitoneal injection N-nitrosodiethylamine (DEN) at day 14, TG and wildtype (WT) mice of both genders were sacrificed at different time points (6–13.5 months). Overexpression of SRY in male TG and ectopic expression of SRY in female TG livers promoted DEN-induced hepatocarcinogenesis compared to age- and sex-matched WT. This accelerated tumorigenesis in TG of both genders was a consequence of increased injury and inflammation, fibrosis, and compensatory enhancement in hepatocytes proliferation secondary to activation of downstream targets Sox9 and platelet-derived growth factor receptor α (PDGFRα)/phosphoinositide 3-kinase (PI3K)/Akt and c-myc/CyclinD1. In conclusion, activation of SRY and its downstream Sox9 and PDGFRα pathways are commonly involved in male hepatocarcinogenesis, which provides novel insights into gender disparity and sex-specific therapeutic strategies of HCC.

Introduction

Hepatocellular carcinoma (HCC) is the second most common cause of cancer-related death all over the world and responsible for 782,500 new cases and 745,000 death annually [1,2]. HCC is prevalent, but different in disease incidence geographically mostly related to distribution of viral hepatitis. Notably, sex is also an influential factor in HCC incidence and mortality [1–3]. Men are about three to six times more likely to develop HCC than women [1,2]. A similar or even more pronounced gender disparity has also been observed in rodent HCC models [3,4]. In fact, an individual's sex is a key factor affecting the risk, aggressiveness, treatment responses and prognosis of cancer [5]. However, the molecular mechanism of this observed disparity is poorly understood.

The liver is a sexually dimorphic organ showing gender difference in gene expression, mitochondrial function, microsomal enzyme activity, membrane lipid composition and immune response [6–8]. Therefore, the sex-biased molecular signatures

List of abbreviations: HCC, hepatocellular carcinoma; TG, transgenic; WT, wildtype; SRY, sex-determining region on Y chromosome; DEN, N-nitrosodiethylamine; PDGFRα, platelet-derived growth factor receptor α; PI3K, phosphoinositide 3-kinase; RBMY, the RNA-binding motif gene on the Y chromosome; TSPY, testis-specific protein Y-encoded; HMG, high-mobility group; PCR, polymerase chain reaction; WB, western blot; IHC, immunohistochemistry; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling; LW/BW, liver weight/body weight ratio.

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might be the major reason for the gender inequalities in HCC development, which suggests a pressing need to develop sex-specific treatment strategies [4–6,9,10]. However, knowledge about the difference of men and women in HCC incidence and mortality at molecular level is very limited. Sex hormones and their receptors have been substantially investigated to identify the cellular and molecular mechanisms contributing to male predominance of HCC. However, normal human liver and HCC express both estrogen and androgen receptors, and administration of either estrogens or androgens in animal models induces hepatocyte proliferation and HCC development [6,11]. Moreover, inhibition of either estrogen or androgen signaling shows no benefits in patient survival [12]. Therefore, there is no robust evidence to consider HCC as a hormone-responsive tumor [12].

Dysregulation of Y chromosome-specific genes has been found in male HCC, e.g. the RNA-binding motif gene on the Y chromosome (RBMY) and testis-specific protein Y-encoded (TSPY) [10,13]. Although only a small number of protein-coding genes are present in the male-specific region of Y chromosome, the impact of Y chromosome genes on human HCC are largely unknown, due to lack of *in vivo* models [14]. Sex-determining region on the Y chromosome (SRY) is a key regulator determining differentiation of the embryonic bipotential embryonic genital ridges towards testis commitment [15]. Therefore, SRY is recognized as a male specific gene highly expressed in male genital tissues. Interestingly, SRY is also normally expressed in other organs and tissues of males including the liver, implying its pathophysiological functions specifically in males, the mechanism of which, however, remains unclarified [16,17]. A few very recent *in vitro* studies have shown that SRY might be an oncogene and a cancer stem cell promoter in HCC of males [18,19]. However, whether SRY expression in liver will necessarily promote hepatocarcinogenesis remains unknown.

Herein, we report the generation and characterization of hepatocyte-specific SRY overexpressing transgenic mice. And overexpression of SRY in male and ectopic expression of SRY in female mice led to more susceptibility to chemical-induced HCC compared to their respective wildtype counterparts. The accelerated tumorigenesis of in TG was associated with increased inflammation, injury, fibrosis, along with compensatory cell proliferation that mediated by activation of Sox9 and PDGFRα/PI3K/Akt pathways.

Materials and methods

Albumin/SRY expression constructs

An approximately 1.2-kilobase mouse SRY gene was amplified. The cDNA was inserted into the *Bam*HI sites of an albumin promoter/enhancer-driven expression vector (Cyagen, Guangzhou, China) to generate the transgenic pRP.ExSi-Albumin-SRY plasmid (Supplementary Table 1).

Generation and genotyping of Albumin/SRY transgenic mice

The previous described construct was linearized and microinjected into C57BL/6 × SJL hybrid mouse eggs to create transgenic mice by Cyagen Biosciences Corporation (Guangzhou, China). The transgenic mice were identified by polymerase chain reaction (PCR) analysis using tail genomic DNA digestion. The primers used for the transgene were as follows: F1: 5′-AAACATACGCAAGGGATTTAGTCA-3′, and R1: 5′-TGTGACACTTTAGCCCTCCGAT-3′. For the primers used for internal control, F1: 5′-CAACCACTTACAAGAGACCCGTA-3′, and R1: 5′-GAGCCCTTAGAAATAACGTTCACC-3′. The internal control PCR targets the endogenous mouse Rgs7 (G protein signaling 7) locus. The transgene and internal control PCR products size were 456bp and 632bp, respectively. Five independent transgenic founder F0 mice (3 males and 2 females) with robust SRY expression were bred with C57BL/6 wild-type mice (WT) to produce F1 heterozygous mice and then bred further to obtain TG mice. The TG mice including both male and female were subject to further studies in comparison with age- and sex-matched WT mice. Animal care and animal procedures were in accordance with the governmental and university guidelines.

Treatments and tissue collection

14-day-old Male TG and WT mice, and female TG and WT mice (20–25 g) were injected intraperitoneally with N-diethylnitrosamine (DEN, Sigma-Aldrich, Inc.) at a dose of 20 μg/g body weight. After DEN exposure, male mice were sacrificed at age of 8 months, 10 months, and 12 months, while female mice were sacrificed at age of 10 months and 13.5 months. Liver tissues with tumor were harvested and fixed in 10% buffered formalin and used for paraffin embedding and sectioning. Only tumor tissues were enucleated and frozen in liquid nitrogen for protein analysis.

Additional methods are provided in the Supporting Information.

Statistical analysis

All experiments were performed three or more times and representative data is presented. Tumor incidence was compared by Chi-square test or Fisher's exact test. Tumor size was expressed as averages ± standard error, and compared for statistical significance by Student *t* tests. Statistical analysis was performed using SPSS 20 for Windows (SPSS, Inc., Chicago, IL). Two-tailed P value of <0.05 was considered statistical significant.

Results

Activation of SRY in human HCC tissue

Expression of SRY was detected in two independent cohorts of patient HCC and paired normal liver tissues by immunohistochemistry (IHC) and western blot (WB), respectively. In the 46 pairs of HCC samples (Supplementary Tables S1) and 33 (71.7%) were from males and 13 (28.3%) were from females. SRY was faintly or moderately expressed cytoplasm of in male hepatocytes but negative in female (Fig. 1A and B). Notably, more than one half of male HCC (18, 54.5%) displayed strongly nuclear and cytoplasmic SRY immunoreactivity, while other 10 (30.3%) showed increased cytoplasmic SRY, compared to paired adjacent liver tissues (Fig. 1A and B). Interestingly, in female HCC samples, 2 (15.4%) showed nuclear and/or cytoplasmic SRY expression, while the rest 11 HCC, and all the paired 13 normal liver tissues, were negative for SRY immunostaining (Fig. 1A and B).

Additional 35 pairs of HCC and normal liver tissues were subject to protein assay by WB. 26 out of 31 (83.9%) male HCC showed increased SRY protein expression than paired normal liver tissues (Fig. 1C). Only 1 out of 4 (25%) female HCC showed faint SRY expression (Fig. 1C). This novel observation suggests that SRY is commonly overexpressed and activated in male HCC, which might be a critical oncogene in promoting male-specific hepatocarcinogenesis.

Increased and ectopic expression SRY protein in male and female TG mice

To investigate the potential role of SRY in HCC development, hepatocyte-specific SRY overexpression TG mice were generated by injection of the albumin/SRY construct into the blastocysts (Fig. 2A). And 10 (7 males and 3 females) out of 60 pups were positive for transgene expression by PCR as F0 founders (Fig. 2B). Five independent transgenic founder F0 mice (3 males and 2 females) with robust SRY expression were bred with C57BL/6 WT to produce F1 heterozygous mice and then bred further to obtain TG mice for further studies.

The TG mice were identified by real time polymerase chain reaction (PCR), WB and IHC. As shown in Fig. 2C and D, SRY mRNA was slightly expressed in male WT mice but negative in female WT mice (Fig. 2C and D). As expected, SRY mRNA was highly expressed in male and female TG mice (Fig. 2C and D). At protein level, expression of SRY and its direct target gene Sox9 were significantly increased in TG versus WT mice of both genders (Fig. 2E). Morphologically, SRY was predominantly expressed in cytoplasm of male WT livers, but strongly expressed in cytoplasm and sparsely in nucleus of male TG livers (Fig. 2F). The female TG livers also showed strong expression of SRY in cytoplasm, in contrast with null expression in WT mice. However, liver weight/body weight ratios (LW/BW) were not significant different between TG and WT mice of both genders (data not shown). These findings demonstrate